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The Phagocyte Function during Multifactorial Military Stress, and Neuroendocrine Interactions with Phagocytes

P. Wiik, P.K. Opstad and A. Bøyum
Norwegian Defence Research Establishment
P.O. Box 25
N-2007 Kjeller
Norway

SUMMARY

The huminal-amplified chemiluminescence response of granulocytes to serum opsonized zymosan particles *ex vivo*, was investigated during a ranger training course lasting for 7 days with continuous strenuous physical activities, calorie and sleep deprivation. A priming for accentuated chemiluminescence response was observed during the first days the course with a maximum increase on Day 3 (+ 35 % of control levels). Thereafter, a reduction to below control values was observed, minimum value was observed on Day 7 (-28 %). One group (N = 8) receiving 6000 kJ/24 h of energy, showed a more pronounced priming during the first days compared to the other group receiving an average of 1000 kJ/24 h (maximum +57 % versus +21 %), and less reduction of the chemiluminescence compared to control on the following days. These data indicate that extreme physical activity for up to a few days primes the production of reactive oxygen species in granulocytes, while activity for a longer time results in a downmodulation.

INTRODUCTION

The neutrophil granulocytes provide the first line of defence against invading micro-organisms but activated granulocytes may also contribute to the damage of normal tissue during inflammation. Granulocytes constitute more than 50% of the leucocytes in peripheral blood, and exhibit a high degree of turn-over with a half life of less than 24 hours. Upon infection or inflammation, granulocytes react with chemotaxis towards e.g. formylated bacterial peptides and complement fragments, and particulate stimuli may be phagocytosed after adhesion to granulocyte surface receptors for complement fragments and for the Fc-portion of immunoglobulins. After phagocytosis, killing and digestion of the ingested material are provided for by production of reactive oxygen molecules and release of proteolytic enzymes into the phagolysosome particle. Reactive oxygen molecules are produced by reduction of oxygen by a membrane bound enzyme (NADPH-oxidase) giving oxygen anion radical as the primary product, and other reactive oxygen molecules and oxygen radicals (like hydrogen peroxide, hydroxyl radical, hypochlorite and chloramines) are formed in spontaneous or enzymatic processes (Babior 1984).

It is often assumed that moderate physical activity stimulates the immune system, while more extreme exercise in terms of intensity is associated with immunosuppression (Fitzgerald 1988, Sharp and Koutedakis 1992). Exercise is known to strongly increase the number of granulocytes in peripheral blood due to the effects of catecholamines and cortisol (Loeper and Crouzon 1904, Bishop et al. 1968, Weicker and Werle 1991, Hack et al. 1992, Gabriel et al. 1992). For granulocyte function, however, quite variable effects of exercise have been reported. In one study, granulocytes from top athletes were less adherent, and exercise to exhaustion reduced the adherence and bactericidal capacity significantly more than in controls (Lewicki et al. 1987). In another study, no difference between athletes and controls were observed in phagocytic capacity and production of reactive oxygen species (Hack et al. 1992). In that study, a small reduction in the production of reactive oxygen species was observed immediately after exhaustive exercise. However, after 24 h of recovery, a significant increase both in the production of oxygen species and in the phagocytic capacity was observed (Hack et al. 1992). Intense (interval) running for up to several hours has been shown to result in post-exercise activation of granulocytes as indicated by degranulation and increased Fc- and C3bi-receptor numbers

(Gray et al. 1993) confirming the findings of Kokot et al. (1988) who found degranulation and reduced chemiluminescence to the phorbol ester PMA after (intense) 10 000 m running.

Little information is available about how granulocyte function is changed during continuous physical activity lasting for more than 2-3 hours. We therefore wanted to study the granulocyte function during a ranger training course lasting for 7 days with continuous, moderate exercise.

SUBJECTS AND METHODS

Subjects and blood sampling. As a part of their training program, the cadets of the Norwegian Military Academy take part in a ranger training course lasting for 7 days. During this course, the cadets are exposed to continuous physical activity corresponding to about 35% of their maximum oxygen uptake (VO_{2max}) around the clock or an energy consumption about 35 000 kJ/24 h. Cadets received no calories on Days 1-2, about 3000 kJ on Day 3, and about 4000 kJ on Day 4, no food on Day 5, 400 kJ on Day 6 and no calories on Day 7. The cadets were allowed no organized sleep during the course, but got short periods of sleep between activities estimated to a total of about 3 h.

Sixteen cadets were randomly selected to participate in the scientific part, and all volunteered. All cadets were males between 20-30 years of age, all exercised regularly, were in good health and used no regular medication. Each day, at 08:00 a.m., venous blood was drawn with EDTA an anticoagulant. The participants were randomly selected into two groups. Group 1 (N = 8) got no extra food, while Group 2 (N = 8) received additional 5000 kJ/24 h of energy throughout the course.

Separation of granulocytes from venous blood. EDTA-blood was mixed with 10% (v/v) of 6% (w/v) dextran in 0.9% (w/v) NaCl. After sedimentation, the leucocyte-rich plasma was layered onto Lymphoprep^R (Nycomed, Oslo, Norway) and centrifuged (15 min, 600 x g) to obtain suspensions of granulocytes (Bøyum 1968). The granulocyte suspension was incubated with 0.83 % (w/v) NH_4Cl to induce hypotonic lysis of the contaminating erythrocytes and was then washed twice in 0.9 % NaCl (400 x g, 7 min, 4°C). Then, granulocytes were suspended in Earle's Balanced Salt Solution (EBSS) without phenol red and supplemented with 50 mmol/l HEPES.

Opsonization of zymosan and the chemiluminescence assay. The procedure was carried out as previously published with small modifications (Wiik 1989). Serum opsonized zymosan was produced by incubation (37 °C, 30 min) of 1 ml of 20 mg/ml zymosan with 5 ml of mixed serum from 6 healthy donors. The opsonized zymosan was then washed twice in sterile water and diluted (2.5 mg/ml) in EBSS.

Opsonized zymosan (0.5 mg/ml) was used to activate the granulocytes (0.5×10^6 cells) and 0.1 mmol/l luminol (5-amino-2,3-dihydro-1,4-phthalazindione; Sigma, St. Louis, MO, USA) was added to a total volume of 0.25 ml. Measurement was done in triplicate, and each sample was measured every 5 minutes for 25 minutes. All buffers and reagents were from the same batch, and care was taken to keep all procedures constant on each day during the course.

Luminol is converted to an excited amonophthalate ion in the presence of oxidizing compounds, and this reaction emits blue light which was measured at 425 nm in a LKB-Wallac1251 luminometer. The chemical basis of the chemiluminescence reaction is not known in every detail, but superoxide anion and the myeloperoxidase product hypochlorite (HOCl) are necessary for generating luminol amplified chemiluminescence (Dahlgren et al. 1991).

Cortisol. Plasma cortisol was measured with radioimmunoassay (RIA) kits from ImmunoDiagnostic Systems Limited, Bolden, UK.

Calculations and statistics. Results are presented as mean (with SD) of N determinations. For analysis of the chemiluminescence data, maximum control value for each person was set to 100%, or given as absolute numbers. Statistical analyses were performed with SPSS^R software program. The statistical significance of

differences was tested by analysis of variance (repeated measures design) with *Day* as the repeated within subject factor. For the chemiluminescence also another within subject factor (*kin*) was included in the model to represent the activity at the five different time points after activation. For testing of *group effects*, control values were included as covariates. Correlations were calculated by the Pearsons product-moment formula.

RESULTS

The numbers of peripheral blood leucocytes and granulocytes during the ranger training course are shown in Fig. 1. An overall increase in the total number of leucocytes ($p < 0.001$) as well as granulocytes ($p < 0.001$) was observed, the maximum increase from control (C1) was observed on Day 1. After this initial maximum, a decrease was observed, but higher leucocyte(s) counts than control levels were observed throughout the course.

An overall significant effect of additional food supplement (Group 2) was observed in the leucocyte and granulocyte counts in peripheral blood during the course (*group \times day* interaction; $p \leq 0.05$) (Fig. 1). The strong increase to Day 1 was similar in the two groups, while Group 2 had somewhat lower leucocyte counts during the rest of the course.

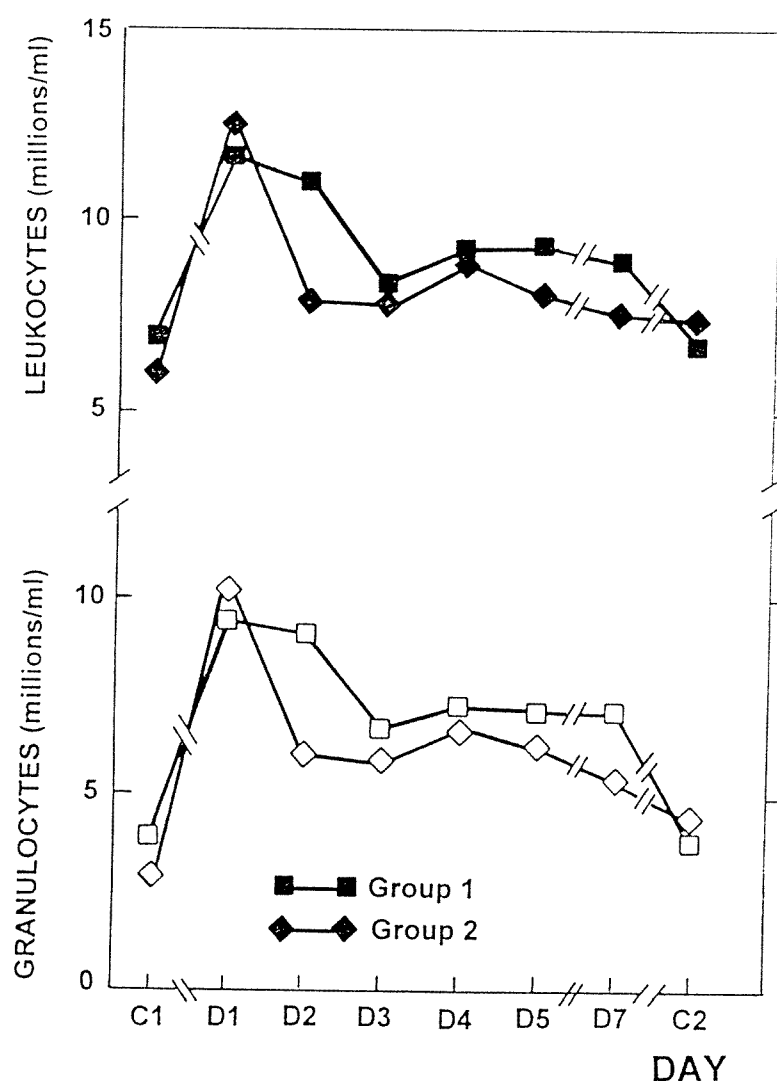


Fig. 1. The total numbers of peripheral blood leucocytes (upper) and granulocytes (lower) are shown in the control situation (C1 and C2) and on different days (D1 to D7) during the ranger training course. Subjects were exposed to continuous exercise around the clock, with energy expenditure about 35 000 kJ/24. Group 1 (N = 8) received an average of 1000 kJ/24 h of energy while Group 2 (N = 8) received 6000 kJ/24.

The chemiluminescence response to serum opsonized zymosan on different days is shown in Fig. 2. There was an overall difference between days ($p < 0.001$). During the first days an increase was observed and the maximum chemiluminescence response was on Day 3 ($p = 0.044$).

The effect food supplement on granulocyte chemiluminescence during the ranger training course is shown in Fig. 2. There was an overall significant effect of additional food supplement on granulocyte chemiluminescence (*group \times day \times kin* interaction, $p = 0.006$). In Group 2 receiving 1000 kJ/24 h, the initial increase was larger (Day 3, $p = 0.047$), and, although not significant, the reduction seemed to be somewhat less pronounced on Days 4-5 for Group 2 while no difference between the Groups was observed on Day 7.

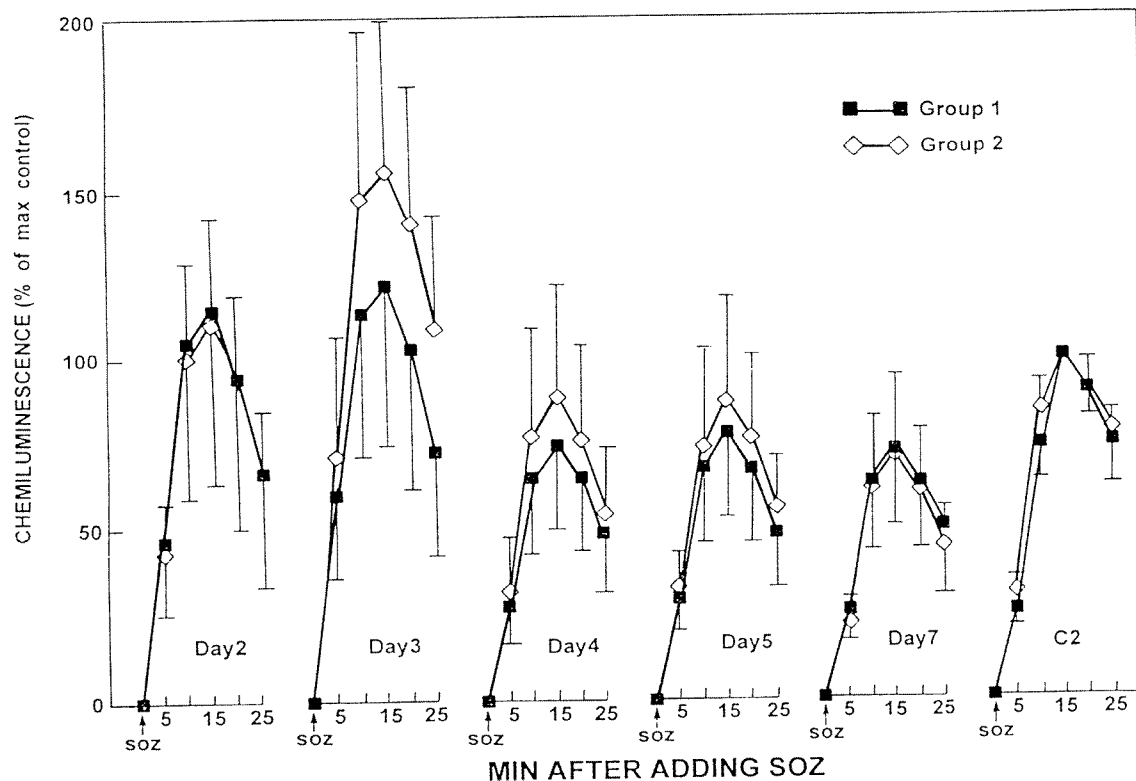


Fig. 2. The luminol-enhanced granulocyte chemiluminescence response (with SD) to serum opsonized zymosan (SOZ) at different days during. Groups as in Fig. 1. Results are expressed as per cent (with SD, N = 8) of max control (C2) value.

Serum cortisol concentrations are shown in Fig 3. Cortisol increased significantly ($p < 0.001$) to a maximum (800 nmol/l) on Day 1 and then submaximal levels (700-800 nmol/l) were observed until Day 6 when a drop to normal values was observed.

The effect of food supplement on serum cortisol levels throughout Course A is shown in Fig. 3. There was an overall difference between the groups (*group \times day interaction*, $p < 0.001$). For Group 1 (which did not receive additional food), high serum cortisol levels were observed on Days 2-6 (730-800 nmol/l) with a maximum on Day 4 (800 nmol/l). In contrast, the cadets receiving extra calorie supplement (Group 2) showed a stronger and earlier increase from control with a maximum on Day 1 (920 nmol/l), then moderate values on Days 2-3 (740 and 790 nmol/l), and normal plasma cortisol levels on Days 4-7.

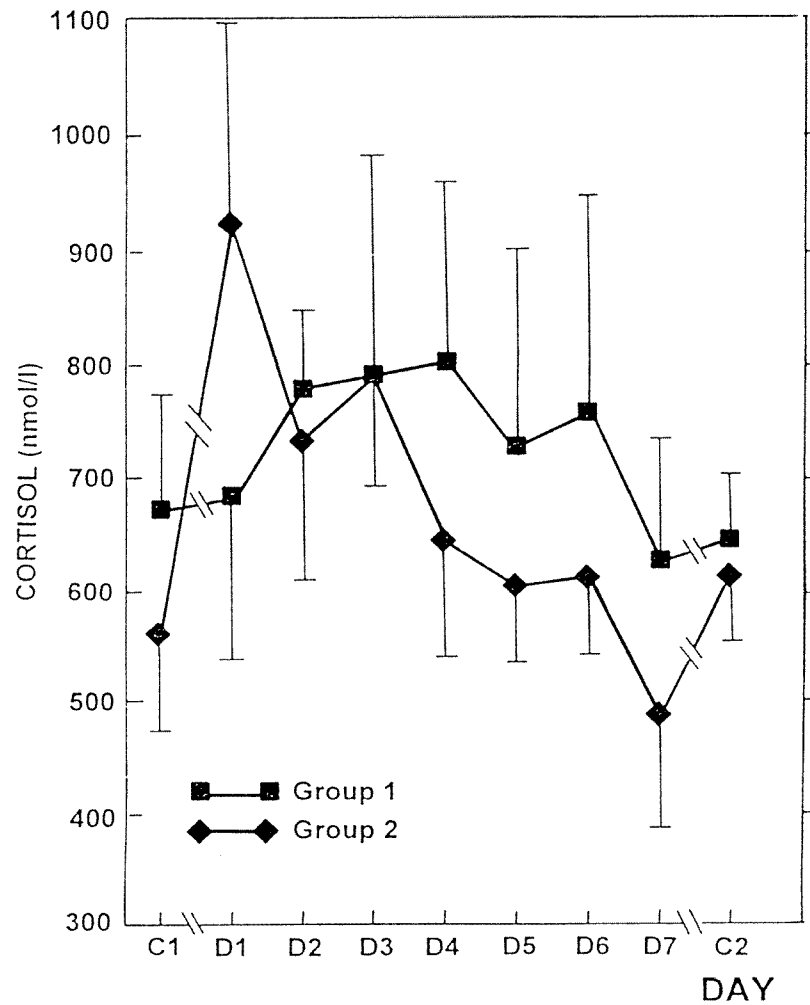


Fig. 3. Plasma concentration of cortisol (with SD) in the control situation (C1 and C2) and at different days during the ranger training course. Groups as in Fig. 1.

Correlation of cortisol with granulocyte numbers and chemiluminescence is shown in Table 1. There was a significant negative correlation between serum levels of cortisol and chemiluminescence in the control situation as well as during the training course. However, the *change* from control to the different days did not correlate for cortisol and chemiluminescence (data not shown). For serum cortisol and the number of granulocytes, a significant positive correlation was observed on Day 1 (Table 1) when the granulocyte number was maximal, but not on the other days (data not shown). The *change* from control to the different days correlated for Day 1 ($r=0.49$, $p=0.05$), not for the other days (data not shown).

Table 1. Correlation of serum cortisol with SOZ-activated granulocyte chemiluminescence and with peripheral blood granulocyte numbers on different days during the course

	Day 2	Day 3	Day 4	C2
Chemiluminescence (15 min value)	-0.55**	-0.58**	-0.37	-0.63**
Granulocyte numbers	0.13	0.30	0.13	0.34

** $p < 0.01$

After the observed increase in chemiluminescence during the first days, a reduction to below control values was observed. Significantly lower values than control were found on Days 4, 5 and 7 ($p < 0.05$).

DISCUSSION

This study demonstrates a biphasic granulocyte response to exercise: During the first 1-3 days of continuous physical activity an *in vivo* priming of peripheral blood granulocytes was taking place, which is in accordance with e.g. Gray et al. 1993. When the physical activity continued for a longer time, a reduction to below control values was observed for the zymosan stimulated production of oxygen species. Furthermore, fasting during the first days (Group 1) was shown to result in lower priming of granulocytes compared to Group 2 with a small energy supply (1000 kJ/24 h).

Reduced granulocyte function may contribute to immune suppression, susceptibility to infections, and may also be part of an overtraining syndrome. On the other hand, the observed priming during the first days may be beneficial for the defence against e.g. bacterial infections, but activated granulocytes are also involved in tissue damage associated with a broad range of pathophysiological conditions. For the musculo-skeletal system this includes exercise-induced muscle-fiber injury, ischemia-reperfusion injury (e.g. joint during exercise) and inflammation (e.g. rheumatoid arthritis, tendinitis) (Babior 1984, Armstrong et al. 1991). However, it is unknown whether granulocytes from blood are representative for granulocytes from local inflammatory processes in the tissues.

The mechanism for the increased chemiluminescence during the first days is not known. However, several factors are known to prime granulocytes, e.g. endotoxin, interleukines, interferons and growth factors (Al-Mohanna and Hallet 1992). The concentration in plasma of several of these factors are changed during and after exercise (Sharp and Koutedakis 1992). During the ranger training course, we have measured a significant increase in the plasma concentrations of GM-CSF (3-4 fold), no change in interleukin 1 and interleukin 2, and a 10-20% reduction in interleukin 6 (Bøyum et al. 1993). These (and other) factors can be involved in the *in vivo* priming mechanism observed.

Cortisol is known to cause immunosuppression, and granulocytes were exposed to high levels of cortisol during several days of the ranger training course as demonstrated in this and previous reports (Opstad and Aakvaag 1981, 1983, Opstad 1991). An inhibitory effect of cortisol on the chemiluminescence is supported by the significant negative correlation observed for cortisol and chemiluminescence. However, other factors also seem to be important for the regulation of granulocyte function during stress, since *change* in cortisol (from control values) did not correlate with *change* in chemiluminescence on the different days.

During an extreme situation like the ranger training course, the subjects are also exposed to significant psychological stress which may as well affect the immune system (Khansari et al. 1990). Further studies are necessary in order to clarify the effect of psychological stress on the granulocyte function.

In summary, this study demonstrates a priming for accentuated production of reactive oxygen species of granulocytes during the first days of continuous physical activity, while physical activity for a longer period of time resulted in suppressed granulocyte function. However, the physiological implications of the presented data are difficult to outline since activation of granulocytes can be beneficial for the host defence against invading microorganisms, but may on the other hand increase the inflammatory damage to normal tissues like e.g. tendons, joints and muscle fibres during exercise.

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